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SHORT COMMUNICATION

A Novel Carbohydrate Addition Site on the Hemagglutinin Protein of a Highly Pathogenic H7 Subtype Avian Influenza Virus[1]

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The highly pathogenic (HP) avian influenza isolate, A/Fowl/Victoria/76 (H7N7), contains two naturally occurring hemagglutinin (HA) variants. The two hemagglutinin proteins differ only in the possession of a potential asparagine-linked glycosylation site at amino acid position 188–190, which is near the proposed receptor binding region of the HA. Expanded virus plaques which possess the addition site exhibit more slowly migrating HA_1 subunits and are significantly more lethal in chickens than those which lack the site. When artificial mixtures of the two variants were inoculated in birds, as few as 1 in 1000 particles containing the glycosylation site was sufficient to exhibit 100% lethality in birds. The data raise the possibility that presence of carbohydrate near the receptor site on the H7 avian influenza virus hemagglutinin may influence virulence.

The avian influenza (AI) viruses provide an excellent model for studying emergence of pathogenic viruses in nature. Strains of the H7 and H5 subtypes, in particular, circulate in wild birds and are prime candidates to act as sources of highly lethal disease when transmitted to commercial poultry (*1*). In 1983, in the northeast United States, one particularly interesting biological scenario unfolded. Following introduction of an H5N2 subtype of low pathogenicity into chickens, apparently from nearby waterfowl, loss of a carbohydrate addition site on the hemagglutinin (HA) was correlated with genesis of virulent virus (*2, 3*). Other studies have further related both virulence and receptor binding to the presence of carbohydrates in AI strains using mutants generated in the laboratory (*4–6*), but studies of natural variants within populations taken from field isolates have not been published.

In late 1975 and early 1976, a highly pathogenic (HP) H7N7 virus was isolated in Australia from a limited outbreak in Victoria (*7*). Since that outbreak the H7N7 subtype has continued to surface in the area (*8, 9*). Complete sequence analysis of the chicken isolates from 1985 (*9*) demonstrated a highly cleavable hemagglutinin and a 98% sequence similarity to isolates from nearby starling populations. The 1985 isolate was also shown to be closely related by partial sequence analysis to the earlier 1975–76 isolates, suggesting the maintenance of the H7N7 in the area.

Interest at our laboratory in emerging subpopulations of HP strains led to analysis of the 1975–76 isolate. It was demonstrated that, as in other H7 isolates analyzed, two hemagglutinin variants were consistently detected in the population (*10*). In this report we have analyzed several expanded plaque populations from this 1975–76 parent isolate and show that two electrophoretic variants of the HA protein differ in only a single amino acid, one of which specifies a glycosylation site at a novel position on the H7 HA_1 subunit.

The H7N7 parent isolate, A/Fowl/Vic/76 (FV76) (*7*), was obtained from Dr. R. Webster (St. Judes Children Hospital, Memphis, TN) and was propagated in BL-3 level containment facilities at Southeast Poultry Research Labs. This isolate has also been variously designated as A/Fowl/Vic/75 and A/Chicken/Victoria/75 in other publications (*11, 12*). The parent was passed once in chicken embryos and inoculated onto chick cells in the presence of 0.1% trypsin. This strain plaques with equal efficiency either in the presence or absence of trypsin, but the enzyme is routinely included when selecting variants from a field population (*13*). Viral plaques were selected at random without regard to size or appearance and amplified by growth for 16 hr in 10-day chick embryos as described (*13*). Amplified plaques of this sort are referred to henceforth as expanded, plaque-origin, populations (EPPs).

[1] No endorsements are herein implied. Brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standards of the products, and the use of the names by the USDA implies no approval of the products to the exclusion of others that may also be suitable.

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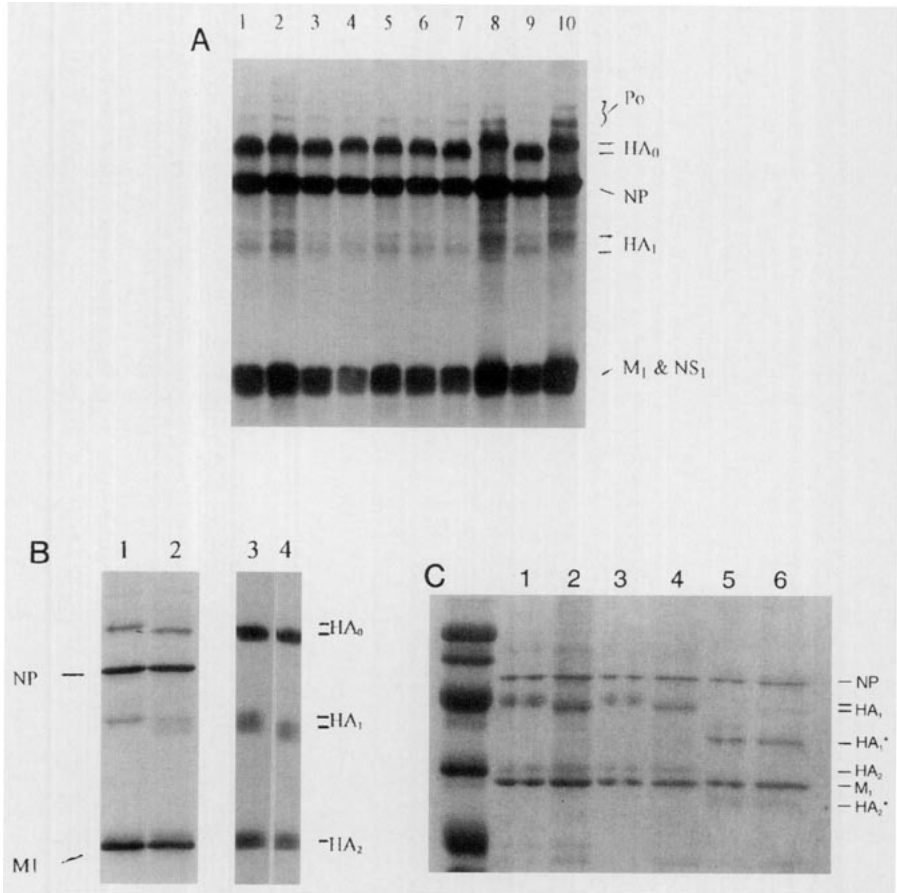

FIG. 1. Identification of variant expanded plaque populations. (A) A typical screening gel of 10 randomly picked and expanded plaques for analysis of viral proteins from chicken cells. Lanes 8 and 10 reveal EPP's with slower migrating uncleaved HA and HA_1 subunits than the remaining EPP's. (B) *Lanes 1 and 2, respectively, SDS–acrylamide gel of* ^{35}S*-radiolabeled cells infected with the prototype d and e expanded plaques populations* (the two which were subjected to nucleotide sequence analysis); lanes 3 and 4, immune precipitates of the cell lysates with H7-specific monoclonal antibody (*10*) to select the hemagglutinin proteins prior to electrophoresis. (C) Coomassie-stained SDS–acrylamide gels of purified viruses from *the d (lanes 1, 3, and 5) and e (lanes 3, 4, and 6) EPPs. Lanes 1 and 2 are untreated, lanes 3 and 4 are treated with PNGase-F glycanase* (Glyko, Inc. Novato, CA) and without disruption of the virions; lanes 5 and 6 are purified viruses first treated with SDS and Triton X-100 then glycanase, following the manufacturers suggested protocol (*18*). The asterisk denotes the deglycosylated forms.

Infected allantoic fluid was extracted and processed for rapid nucleotide cycle-sequence analysis as previously described (*14*). Primers used for PCR and sequence analysis terminated at the following nucleotide positions relative to the positive sense hemagglutinin gene coding sequence: plus sense primers, −60 142, 436, 822, 1380; minus sense primers, 284, 511, 725, 803, 1036, 1360, +48. Sequences were determined using an ABI automated sequencer in the PCR-sequence facility at Southeast Poultry Research Laboratory. Nucleotide and amino acid sequences were collated, compared, and manipulated using Intelligenetics' Geneworks software package. Nucleotide sequences for *d* and *e* were deposited with the EMBL bank, under Accession No. Z47199.

Allantoic fluid harvested from eggs infected overnight with plaques was used as inoculum to infect primary chicken embryo cell cultures. Cells were infected and labeled with methionine as previously described (*15*). Proteins were solubilized and analyzed in denaturing, discontinuous SDS–polyacrylamide gels (*16*) to identify variant EPPs.

Expanded virus plaques were titrated in embryos by standard techniques and diluted in BHI broth containing antibiotics. Equal amounts of virus were inoculated into 4-week-old white rock chickens from the SEPRL-specific pathogen-free flocks as previously described (*17*). All experiments were carried out under BL-3AG level conditions in the high containment animal facilities at SEPRL.

Several EPPs of the FV76 parent were assayed for relative mobility of the HA protein in SDS–acrylamide gels (Fig. 1A). The parent stock contained at least two populations easily distinguishable on the basis of mobility of the uncleaved HA and the HA_1 subunit. These types of electrophoretic variants have been previously designated (*10*) as "R" (rapid) or "S" (slow). Of some 50 plaques of FV76 analyzed, 7 exhibited the "S" form of hemagglutinin (data not shown). Thus the subpopulation of viruses with a slower migrating hemagglutinin represented 10–

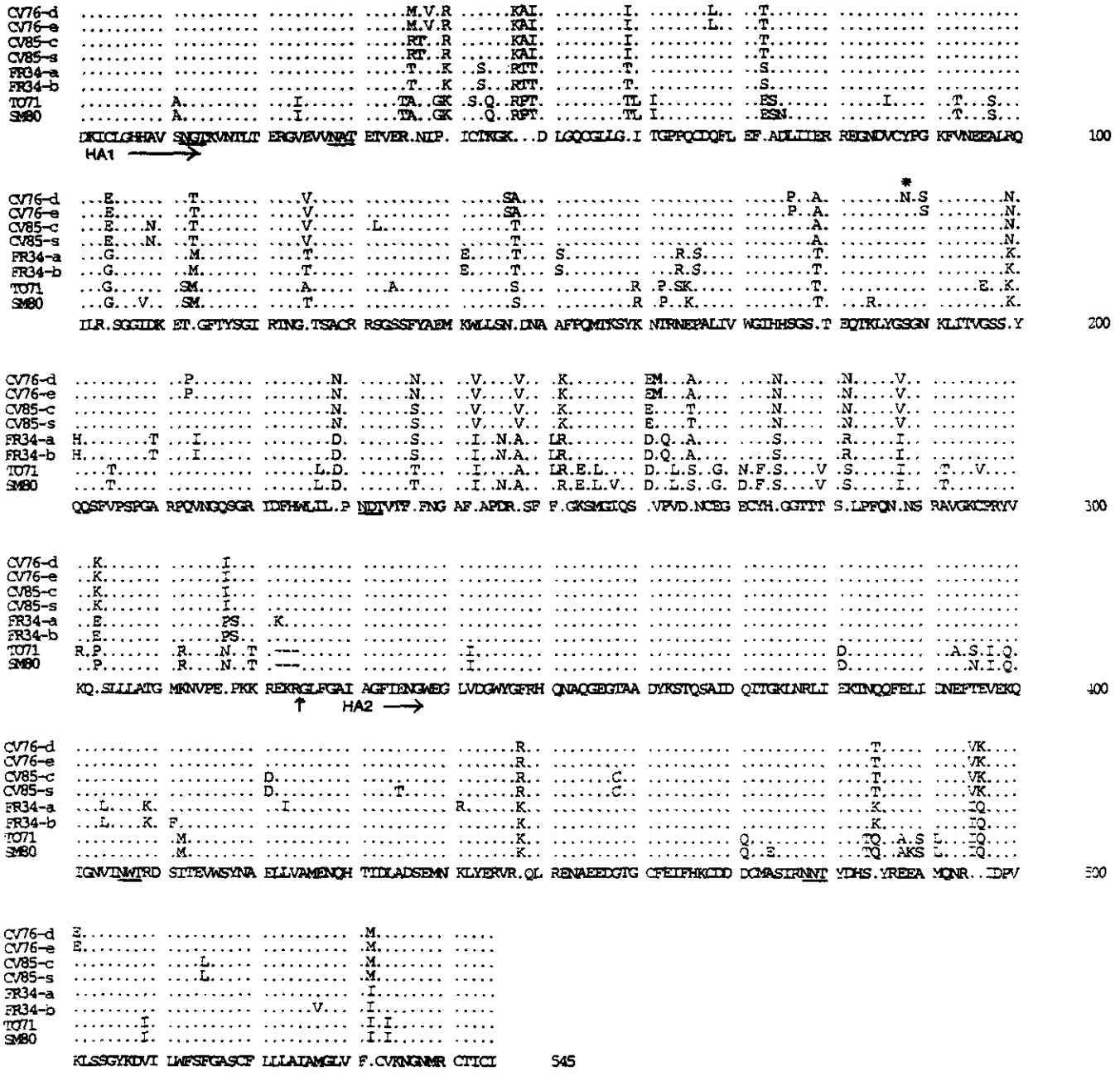

FIG. 2. Complete amino acid sequence of the hemagglutinin proteins of variants *d* and *e* from A/Fowl/Victoria/76 (H7N7), compared with other published H7 sequences. Legend: CV76-d (variant *d*), CV76-e (variant *e*); CV85-c, A/chicken/Victoria/85 H7N7 (*9*); CV85-s, A/starling/Victoria/85 H7N7 (*9*); FR34-a, A/fowl/Rostock/34 H7N1 (*33*); FR34-b, A/Fowl/Rostock/34 H7N1 (*34*); TO71, A/Turkey/Oregon/71 H7N3 (*35*); SM80, A/Seal/Massachusetts/80 H7N7 (*36*). The cleavage site is denoted with a vertical arrow, conserved glycosylation sites are underlined. An asterisk marks the lone amino acid difference between the *d* and *e* variants.

20% of the parent population as originally provided to SEPRL. Two representative "S" and "R" EPPs were selected to analyze in this study. They were designated as *d* and *e*, respectively. These two EPPs were further analyzed using monoclonal antibody precipitation of infected cell lysates (Fig. 1B), which clearly demonstrated that migration differences were localized to the HA_1 subunit of the HA protein. The migration differences were also shown to be due to glycosylation of the HA protein. Treatment of purified virus with recombinant *N*-glycanase (PNGase-F, Glyko, Inc., Novato, CA) under conditions in which the enzyme is active (*18*) resulted in the variant HA proteins migrating at the same rate (Fig. 1C). Inability to recover sufficient quantities of intact purified HA protein by standard techniques precluded an analysis of partial proteolytic digests of the HA to further localize the glycosylation site. However, the coding regions of the HA genes of the two variant EPP's (*d* and *e*) were se-

TABLE 1

Lethality of A/Fowl/Victoria/76 in Standard Pathotyping Assays

		Expanded plaque populations								Mixture of 7:8	
	Parent	1	2	3	4	5	6	7	8	1:10	10:1
Lethality[a]	8, 8, 8, 8	2, 2, 1	8, 8, 8	3, 1, 0	8, 8, 8	3, 4	8, 8	1, 2	8, 8	8, 8	8, 8
Type of HA[b]	Mix	R	S	R	S	R	S	R	S	—	—
AA at 188[c]	Ser	Ser	Asn	Ser	Asn	Ser	Asn	Ser	Asn	—	—

[a] Each number represents the number of dead 4-week-old chickens of eight inoculated in a separate cage.

[b] HA protein was designated as S (slowly migrating) or R (rapidly migrating) based upon electrophoretic analysis of labeled chick cell lysate infected with allantoic and harvested at 6 hr p.i.

[c] The amino acid at position 188 in the HA_1, as determined by RT-PCR nucleotide sequence analysis of extracted allantoic fluid.

quenced in their entirety to identify the glycosylation sites.

Figure 2 presents a comparison of the amino acid sequences of the *d* and *e* variants with those of six other H7 avian influenza strains for which a complete HA sequence has been published. Only one nucleotide difference between the variant populations was identified, resulting in a single amino acid difference. At position 188, an asparagine rather than a serine was found in the *d* HA_1, creating the glycosylation signal Asn-Gly-Ser. It is most likely that glycosylation of this site is responsible for the obvious difference in relative mobility of the HA_1 since that is the only structural difference found. Glycosylation of this site would place a carbohydrate side chain near the proposed receptor binding site in the globular tip of the HA trimer.

Of significant note also in Fig. 2 is the fact that the 1975 isolate differed by 17 amino acids from the later 1985 H7N7 isolate, suggesting, as earlier nucleotide sequence analysis had (*9*), that the two were closely related strains. The data also showed several regions of identity between the 1976 and 1985 isolates, not found in the Rostock strain or the North American H7 strains. The Asn-Gly-Ser at position 188 site was not present in the later 1985 isolate nor was it identified in any other H7 HA thus far published. The presence of this unique potential glycosylation signal at this site on the H7 HA led us to determine whether single plaque-generated populations containing the site differed in their virulence for chickens.

Current regulatory guidelines state that AI viruses which are lethal to more than six of eight 4-week-old chickens in 10 days or less should be classified as highly pathogenic (*19*). Taken as a whole, the pathotyping assays demonstrated that of a total of five FV76 EPPs analyzed with the slower migrating ("S" type) HA's all were 100% lethal in 4-week-old chickens, with a mean death time of 2–2.5 days (Table 1, Table 2, and data not shown). Likewise, the parent isolate was always 100% lethal. Plaques generating populations with no detectable slow-migrating HA by gel analysis, however, yielded variable results. In the assays shown in Table 1, it is seen that variants similar to the *e* prototype population (i.e., lacking the glycosylation site by sequence analysis and protein gel analysis) did not score as highly pathogenic while all of the clones which had the slower migrating HA and the potential glycosylation site killed eight of eight chickens in the pathotyping assays. The parent, of course, which had been previously characterized as highly pathogenic, was lethal to 100% of inoculated birds. Interestingly, mixtures of EPPs were also 100% lethal, even when a nonhighly pathogenic expanded plaque population (7) was in 10-fold excess over the HP EPP (8) in the inoculum.

When sequence analysis of the unselected parent was carried out, the glycosylation addition site was not identified. This was not wholly unexpected as the majority of EPPs derived from the parent migrated as "R" variants (see Fig. 1A and Ref. 10). Subsequent assays revealed at least two EPPs which, by sequence analysis, also did not possess the site but yet were classified as highly pathogenic. This data is explained by Table 2, where several dilutions and mixtures of dilutions of "S" and "R" variant populations were tested in pathotyping assays. As few as 10 ELD_{50} units of a variant possessing the glycosylation site at Asn 188, in the presence of a 10,000-fold excess of variants lacking the site, were sufficient to be 100% lethal, albeit with an increased mean time to death. Thus, very low levels of HA containing the potential glycosylation site in the virus population markedly increases virulence of the population.

This report describes the presence of a glycosylation site heretofore not identified among H7 hemagglutinin proteins. The presence of the site, which is associated with 100% lethality in birds, does not affect the extent or rate of cleavage of the HA into HA_1 and HA_2 (*10*). Thus it differs from the effects described for H5N2 isolates from Pennsylvania in 1983, where a glycosylation site inhibited cleavage (*20*). In addition, the presence of the site is also associated with an increase in hemagglutinating activity, while the replication efficiency in embryos and chicken fibroblast cells is the same for each variant (*10* and unpublished data).

TABLE 2

Chicken Lethality of Mixtures of Virus Subpopulations from A/Fowl/Vic/76

	Concentration of virus (ELD_{50} units inoculated)											
"R" form[a]	0	0	0	0	0	0	10^5	10^5	10^5	10^5	10^5	10^5
"S" form[b]	0	10^1	10^2	10^3	10^4	10^5	0	10^1	10^2	10^3	10^4	10^5
Mortality (in 10 days)	0/8	7/8	8/8	8/8	8/8	8/8	2/8	8/8	6/8	8/8	8/8	8/8
Morbidity	0/8	8/8	8/8	8/8	8/8	8/8	3/8	8/8	6/8	8/8	8/8	8/8
Mean death time	—	4.3	2.5	2.9	2.1	2.3	3.5	4.4	4.7	3.4	3.3	2.8

[a] Titrated, first-passage, allantoic fluid from an expanded plaque population (population 1 from Table 1).
[b] Titrated, first-passage, allantoic fluid from an expanded plaque population (population 2 from Table 1).

Given the differences in virulence and hemagglutinating activity associated with the glycosylation signal, these data argue for a possible role in interaction with cell membranes. Based on the computer-generated three-dimensional structure of the H7 hemagglutinin (*21*), this glycosylation signal is located just adjacent to the proposed receptor binding site (as defined for the H3 hemagglutinin; *22*). It is possible that glycosylation provides a selective advantage for attachment to tissues, infection of which would more likely result in a lethal infection. This could account for the differences in hemagglutinating activity without resulting in differences in infection of cultured fibroblasts or cleavage efficiency. Single amino acid variations have been shown to affect hemagglutination efficiencies in human strains (*23*), and amino acid changes in the hemagglutinin of H5 subtype avian influenza strains have been correlated with altered tissue tropism (*24*). Further studies are in progress to determine the importance of the glycosylation site as it relates to both pathology and to immune responses induced by the virus.

Because of the polygenic nature of expression of virulence by influenza viruses (*25, 26*), the presence of the glycosylation site on the HA gene cannot be *unequivocally* associated with the virulence differences from the present study. Genetic reassortment assays in a heterologous system would provide no advantage over the reassortment occurring within the parent strain itself. Given the high mutation rate for Orthomyxovirus genes including the HA (*27, 28*) and the fact that variations in the mutation rate may occur even between plaques from the same population (*29*), reassortment assays would provide no advantage over the approach of selecting and analyzing expanded plaques from the original parent. Since the alteration is due to a single nucleotide change, the reversion rate back and forth between the two variant forms would preclude deriving a homogenous population with certainty. Furthermore, as shown in Table 2, clearly a small concentration of a population possessing the glycosylation site can dramatically affect the virulence pattern. Thus the data argue strongly, but do not prove, that the potential glycosylation site at position 188 enhances virulence of this strain.

The significance of the presence of these two variants in the field is not entirely clear. A separate H7N7 isolate was recovered from nearby ducks during the 1976 outbreak (*11, 30*); it lacked three of the basic amino acids at the cleavage site and was nonpathogenic (*31*). It is not yet clear, however, what biological relationship that isolate shares with the HP isolate.

These data suggest possible roles for glycosylation sites in expression of virulence in avian strains of influenza. They also illustrate the similarity of the HA protein from AI strains with that of human strains in the variable occurrence of potential glycosylation signals at various nonconserved sites near the tip of the membrane-distal globular region of the HA (*32*). This suggests that, as in the case of human strains, as more avian HA genes are sequenced, significantly more pleomorphism will be encountered in HA structure. More importantly, these secondary and tertiary structures may not be related to cleavage of the HA but may still significantly affect virulence expression. Finally these data illustrate, as in past studies (*10, 14*), the importance of analyzing the entire population of viruses in an AI isolate rather than selecting a single, highly "cloned" population to represent the characteristics of the field population.

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